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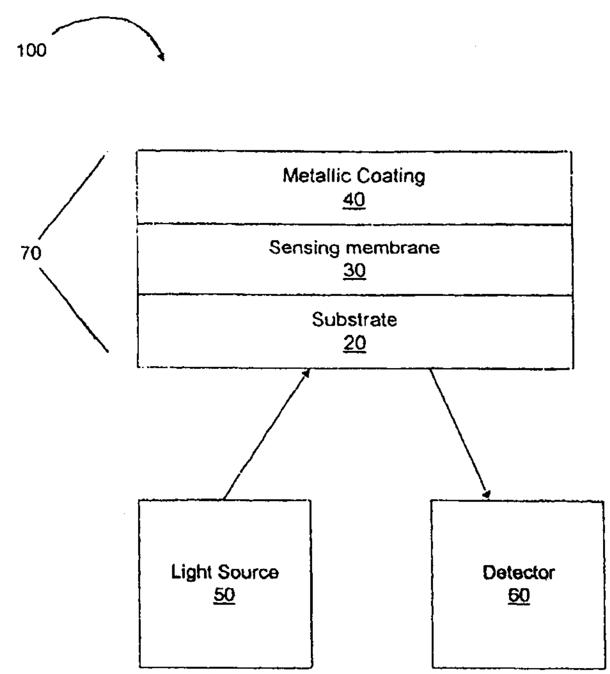
(54) Title: METALLIC OVERCOATING AS A LIGHT ATTENUATING LAYER FOR OPTICAL SENSORS

(57) Abstract

A liquid permeable metallic coating (40) is utilized in conjunction with a fluorescence based optical sensor (70). The metallic coating is deposited directly on, and is in physical contact with, the sensing membrane (30). The metallic coating does not require an intervening substrate layer or other components. When light from a light source (50) is shone through the substantially light transmissive substrate onto the sensing membrane (30), the metallic overcoating (40) reflects back the excitation light as well as the fluorescence light generated by the sensor such that substantially no light reaches the sample where the light may be scattered and/or absorbed by the sample. Accordingly, the accuracy and repeatability of the sensor (70) is improved while the cost and production times associated with manufacturing the sensor are minimized.



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TITLE OF INVENTION

Metallic Overcoating as a Light Attenuating Layer for Optical Sensors

CROSS-REFERENCE TO RELATED APPLICATIONS

Not Applicable

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not Applicable

BACKGROUND OF THE INVENTION

Fluorescent based optical sensors wherein a sensing membrane is layered onto a light transmissive substrate are known. The sensing membrane of the sensor is brought into contact with a sample while an excitation light reaches the sensing membrane through the substrate. The combination of the excitation light, the sensing membrane and a particular analyte will cause the sensing membrane to emit a fluorescing light. The emission signal from the sensing membrane is then detected through the light transmissive substrate from the back side of the sensor. Due to the fact that the sensing membranes of the sensor are quite thin there is a fairly large amount of the excitation light which passes through the sensing membrane and into the sample or into the sample chamber. The light which passes through the sensing membrane may be scattered, absorbed or reflected by the sample or the chamber walls back into and through the sensing membrane. Additionally, the fluorescing signal emitted from the sensing layer, which is indicative of the detection of the amount of the analyte of interest of the sample under test, may also be absorbed, scattered or reflected by the sample back to the detector. The scattering, absorbing or reflecting of the excitation light and the fluorescing light emitted by the sensing membrane can combine to provide a four fold change in the signal between a perfectly reflecting and perfectly absorbing signal, thus severely skewing the detection results of the sensor.

Previous attempts to address this issue of unintended light affecting the results of the sensor include coating the sensing membrane with a support layer material which has been impregnated with a second material, or coating the sensing membrane with a plurality of layers such that the amount of light escaping the sensor into the sample and sample chamber is a very small fraction of the total light directed to the sensor. These attempts utilized a complex chemical process to produce an opaque, chemically permeable multilayered structure which is then laminated onto the sensing membrane. For example, U.S. Patent No. 5,091,800 discloses the construction of an ion permeable cover membrane formed from a cross linked PVOH or cellophane substrate which is stretched onto a form and impregnated with silver, gold or platinum colloidal precipitants through a series of chemical treatments to form the opaque membrane. U.S. Patents 5,081,041 and 5,081,042 disclose the use of an ion permeable cover membrane fabricated from a Dextran or cellulose substrate and impregnated with detergent solvated carbon black. U.S. Patents No. 4,919,891 and 5,075,127 utilize cellulose acetate/acetone mixtures of either copper pthalocyanine or carbon black cast as separate coating membranes. U.S. Patent No. 3,992,158 discloses the incorporation of a separate TiO_2-containing cellulose acetate for opacity or reflectance to be used in absorbance based chemistries on dry slides. Similarly, U.S. Patents No. 4,042,335, 4,781,890, 4,895,704 and EP 0 142 849 B1 disclose the use of light blocking layers incorporating TiO_2 particles for slide based chemistry tests. Such techniques have proven to be complex, labor intensive and expensive, requiring the utilization of multiple components or multiple layers of materials. It would be desirable to provide an inexpensive and simple to produce sensor including a single light attenuating layer of material deposited directly on the sensing membrane which reflects excitation and emission light back into the sensor without the light being affected by the sample while permitting the analyte of interest to freely diffuse through the light attenuating layer and into the sensing membrane.

BRIEF SUMMARY OF THE INVENTION

A liquid permeable metallic coating is utilized in conjunction with a fluorescence based optical sensor. The metallic coating is deposited directly on, and is in physical contact with, the sensing membrane. The metallic coating does not require an intervening support layer of material, or other components. When light from a light source is shone through the substantially light transmissive substrate onto the sensing membrane, the metallic overcoating reflects back the excitation light as well as the fluorescence light generated by the sensor such that substantially no light reaches the sample where the light may be scattered and/or absorbed by the sample. Reflectance from within the sample cavity is also avoided. Accordingly, the accuracy and repeatability of the sensor is improved while the cost and production times associated with manufacturing the sensor are minimized.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

The invention will be more fully understood from the following detailed description taken in conjunction with the accompanying drawings in which:

Fig. 1 is a block diagram of a prior art sensor;

Fig. 2 is a block diagram of a sensor including the metallic coating of the present invention;

Fig. 3A is a graph of test results for the prior art sensor of Fig. 1; and

Fig. 3B is a graph of test results for the sensor including the metallic coating of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

A prior art sensing system 10 is shown in Fig. 1 and includes a sensor 15, a light source 50, and a detector 60. Sensor 15 comprises a light transmissive substrate 20 having a sensing membrane 30 layered thereon. In operation, sensing membrane 30 is brought into contact with a sample (not shown) being tested. The light source 50 provides an excitation light to substrate 20, such as through an optical fiber. Substrate 20 is generally light transmissive, thus the light from light source 50 passes through substrate 20 and falls on sensing membrane 30. Sensing membrane 30, in the presence of the excitation light and in the presence of a particular analyte in the

sample will emit a fluorescing light to a degree defined by the concentration of the analyte in the sample. This fluorescing light provided by sensing membrane 30 will pass through substrate 20 and be detected by the detector 60.

Since sensing membrane 30 is relatively thin, excitation light also passes through the membrane 30 and into the sample. Once the excitation light is received by the sample it may be scattered, absorbed and/or reflected back through the sensing membrane 30, and through substrate 20 to be detected by detector 60. Additionally, the fluorescing light produced by the sensing membrane may also pass through sensing membrane 30 and into the sample where it may be scattered, absorbed and/or reflected. Again, this light may pass through sensing membrane 30 and through substrate 20 where it will be detected by detector 60. Accordingly, the measurement results of the sensor can be skewed greatly.

Referring to Fig. 2 a sensing system 100 is shown. The system 100 includes a sensor 70, a light source 50 and a detector 60. The light source 50 and detector 60 are in communication with the sensor 70 through any suitable means, including a fiber optic channel. Sensor 70 includes a substrate 20, a sensing membrane 30 layered on the substrate 20 and a metallic coating 40 layered on the sensing membrane 30. Substrate 20 may be made of any substantially light transmissive material such as cellulose acetate, cellulose acetate butyrate, polyethylene terephthalate, bisphenol A polycarbonate, polystyrene, polymethyl methacrylate or preferably glass.

The sensing membrane 30 is deposited onto a surface of the substrate 20. The sensing membrane 30 may comprise any material or group of materials formed together which provide a detectable indication in response to exposure to a specific analyte of the sample. In a preferred embodiment the sensing membrane 30 is made of copolymer JB3001/23 which comprises a mixture of ethylhexylmethacrylate, methylmethacrylate and an oxygen sensing dye such as octa-ethyl-Pt-porphyrin (OEP).

The sensor 70 further includes a liquid permeable metallic overcoating 40 which is generally nontransmissive to light. The metallic overcoating 40 may comprise Aluminum, TiO_2 or preferably a Gold Palladium mixture. The metallic overcoating 40 may be deposited onto the sensing membrane 30 by sputter coating,

evaporating or other means, thus no intervening support layer or substrate is required between the metallic coating 40 and the sensing membrane 30.

In operation, metallic coating 40 is brought into contact with a sample (not shown) being tested. Metallic coating 40 is liquid permeable such that the sample can diffuse through metallic coating 40 and contact sensing membrane 30. The light source 50 provides an excitation light to substrate 20. Substrate 20 is generally light transmissive, thus the light from light source 50 passes through substrate 20 and falls on sensing membrane 30. Sensing membrane 30 in the presence of the excitation light and in the presence of a particular analyte of the sample will emit a fluorescing light. This fluorescing light provided by sensing membrane 30 will pass through substrate 20 and be detected by a detector 60.

Since sensing membrane 30 is relatively thin, light also passes through the membrane 30 and onto metallic coating 40. Metallic coating 40 is generally nontransmissive to light and reflects the light back through the sensing membrane 30 without allowing a significant amount of the light to reach the sample where the light can be affected by the sample and be subsequently detected by detector 60. Additionally, the fluorescing light produced by the sensing membrane may also pass through sensing membrane 30 where it will also encounter metallic coating 40. Once again, metallic coating 40 will reflect the light back to the sensor without a significant amount of the light passing through to the sample where the light may be affected by the sample and subsequently detected by the detector 60. Accordingly, the excitation light and fluorescing light are not affected by the sample, thus the sensor provides a much more accurate and repeatable sensing of analytes.

Referring now to Fig. 3A a Stern/Volmer plot of a fluorescence intensity in response to varying levels of oxygen is shown as detected by a prior art sensor. A clear, aqueous buffer solution was plotted (denoted by the squares) as was a sample having twenty three grams per deciliter of total hemoglobin (THb) (denoted by triangles) and a sample having nine grams per deciliter of THb (denoted by circles). As seen from the plot, the samples having different THb levels produced a large difference in fluorescence, which can be attributed to the presence and detection of interfering light, such as excitation light which has been scattered, absorbed and

reflected by the sample as well as fluorescing light which has been reflected, absorbed or scattered by the sample and has been detected by the detector.

Referring now to Fig. 3B it can be seen that the same tests performed using similar solutions with the sensor of the present invention provide a much more uniform response. The sensor here has a liquid permeable metallic coating which has an optical density of approximately 0.893. A Stern/Volmer plot of a fluorescence intensity in response to varying levels of oxygen is shown as detected by the sensor of the present invention. A clear, aqueous buffer solution was plotted (denoted by the squares) as was a sample having twenty one grams per deciliter of THb (denoted by triangles) and a sample having six grams per deciliter of THb (denoted by circles). As seen from the plot, the samples having different THb levels produced a generally uniform fluorescence, which can be attributed to the absence of interfering light, such as excitation light which has been scattered, absorbed and reflected by the sample as well as fluorescing light which has been reflected, absorbed or scattered by the sample. Due to the inclusion of the metallic coating directly on the sensing membrane, very little light passes through the metallic coating and to the sample where in can be reflected, absorbed or scattered and provide interfering light which skews the results.

The incorporation of a metallic coating which is liquid permeable as well as being generally nontransmissive to light provides a substantial improvement in the repeatability of sample testing and for testing a variety of different samples. The metallic coating is applied directly onto the sensing membrane without the use of an intervening support layer or without the use of multiple layers of materials thus providing a cost effective manner of including the metallic overcoating since additional materials and labor are minimized, while performance and reliability are greatly improved.

Having described preferred embodiments of the invention it will now become apparent to those of ordinary skill in the art that other embodiments incorporating these concepts may be used. Accordingly, it is submitted that the invention should not be limited to the described embodiments but rather should be limited only by the spirit and scope of the appended claims.

CLAIMS

We claim:

1. A sensor comprising:

a substrate substantially transmissive to light, having a first side and a second side;

a sensing membrane layered onto the second side of said substrate; and

a liquid permeable, substantially nontransmissive to light metallic coating directly layered on said sensing membrane.

2. The sensor of claim 1 wherein said metallic coating is sputter coated onto said sensing membrane.

3. The sensor of claim 1 wherein said metallic coating is evaporated onto said sensing membrane.

4. The sensor of claim 1 wherein said metallic coating has an optical density of between approximately 0.2 and approximately 0.893.

5. The sensor of claim 1 wherein said metallic coating comprises gold palladium.

6. The sensor of claim 1 wherein said metallic coating comprises aluminum.

7. The sensor of claim 1 wherein said metallic coating comprises TiO_2.

8. The sensor of claim 1 wherein said sensing membrane comprises a mixture of ethylhexylmethacrylate, methylmethacrylate and an oxygen sensing dye.

9. The sensor of claim 8 wherein said oxygen sensing dye comprises octa-ethyl-Pt-porphyrin (OEP).

10. The sensor of claim 1 wherein said substrate comprises glass.

11. The sensor of claim 1 wherein said substrate is selected from the group consisting of cellulose acetate, cellulose acetate butyrate, polyethylene terephthalate, bisphenol A polycarbonate, polystyrene, and polymethyl methacrylate.

12. The sensor of claim 1 further comprising:

a light source in communication with said substrate, said sensing membrane and said metallic coating; and

a detector in communication with said substrate, said sensing membrane and said metallic coating.

13. A sensor comprising:

a substrate substantially transmissive to light, having a first side and a second side; a sensing membrane layered onto said second side of said substrate;

a liquid permeable substantially nontransmissive to light metallic coating layered directly onto said sensing membrane, said metallic coating having an optical density of approximately 0.893;

a light source in communication with said substrate, said sensing membrane, and said metallic coating; and

a detector in communication with said substrate, said sensing membrane and said metallic coating.

14. A method of making a sensor comprising:

providing a substrate substantially transmissive to light, having a first side and a second side;

depositing a sensing membrane onto the second side of said substrate; and

depositing a metallic coating directly on said sensing membrane, the metallic coating being liquid permeable and substantially nontransmissive to light.

15. The method of claim 14 wherein said step of depositing a metallic coating comprises sputtering the metallic coating onto said sensing membrane.

16. The method of claim 14 wherein said step of depositing a metallic coating comprises evaporating the metallic coating onto said sensing membrane.

17. The method of claim 14 wherein said step of depositing a metallic coating comprises depositing a metallic coating having an optical density of between approximately 0.2 and approximately 0.893.

18. The method of claim 14 wherein said step of depositing a metallic coating comprises depositing a coating selected from the group consisting of gold palladium, aluminum and TiO_2.

19. The method of claim 14 wherein said step of depositing a sensing membrane comprises depositing a mixture of ethylhexylmethacrylate, methylmethacrylate and an oxygen sensing dye.

20. The method of claim 14 wherein said step of depositing a sensing membrane comprises depositing a mixture of ethylhexylmethacrylate, methylmethacrylate and octa-ethyl-Pt-porphyrin (OEP).

21. The method of claim 14 wherein said step of providing a substrate comprises providing a substrate selected from the group consisting of glass, cellulose acetate, cellulose acetate butyrate, polyethylene terephthalate, bisphenol A polycarbonate, polystyrene, and polymethyl methacrylate.

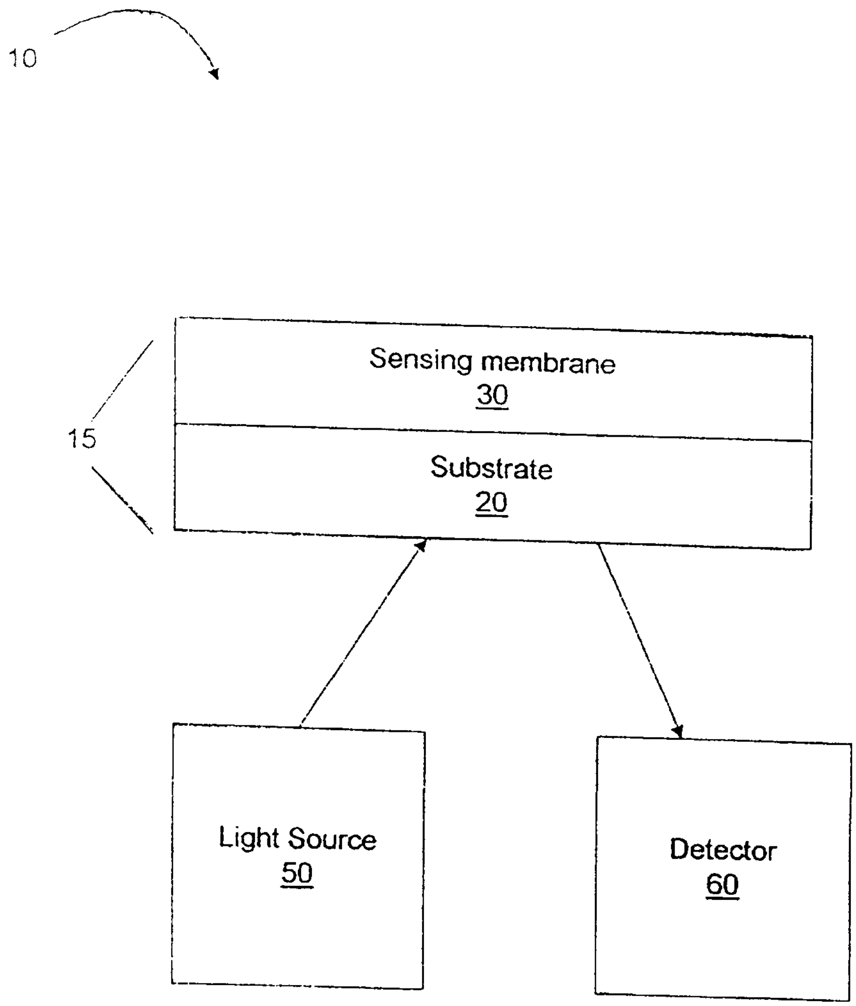

10
15
Sensing membrane
30
Substrate
20
Light Source
50
Detector
60

FIG.1

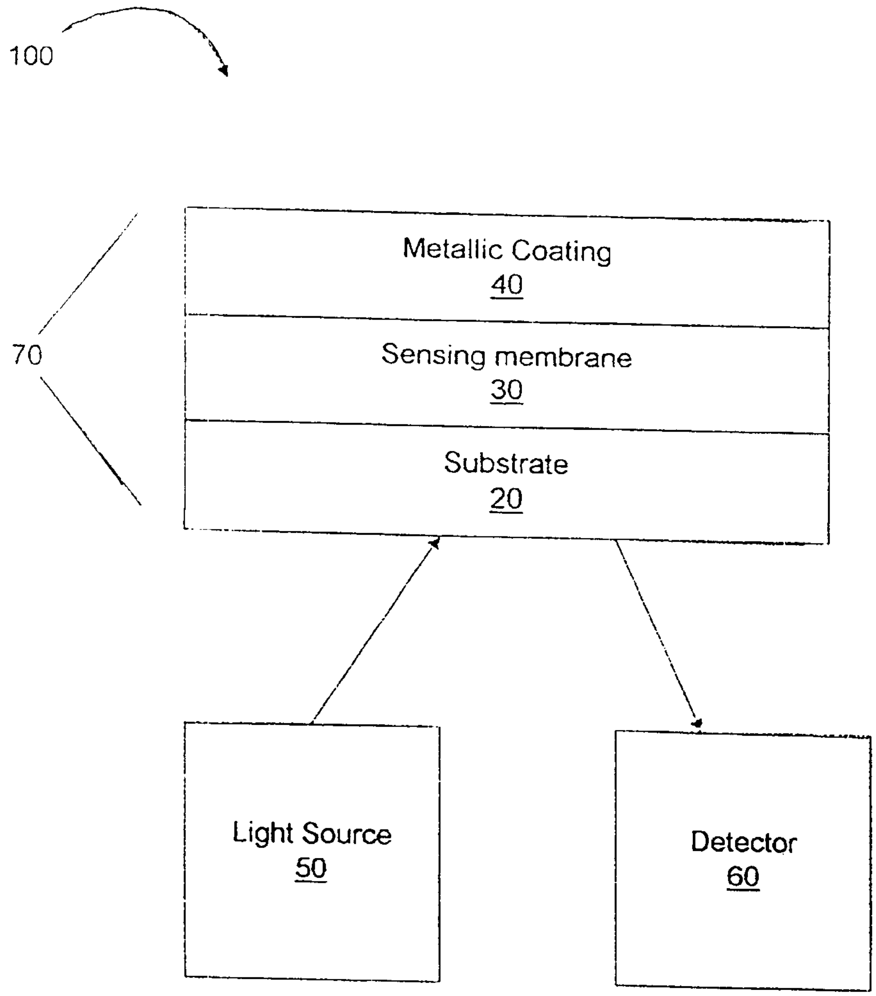

100
70
Metallic Coating
40
Sensing membrane
30
Substrate
20
Light Source
50
Detector
60

FIG.2

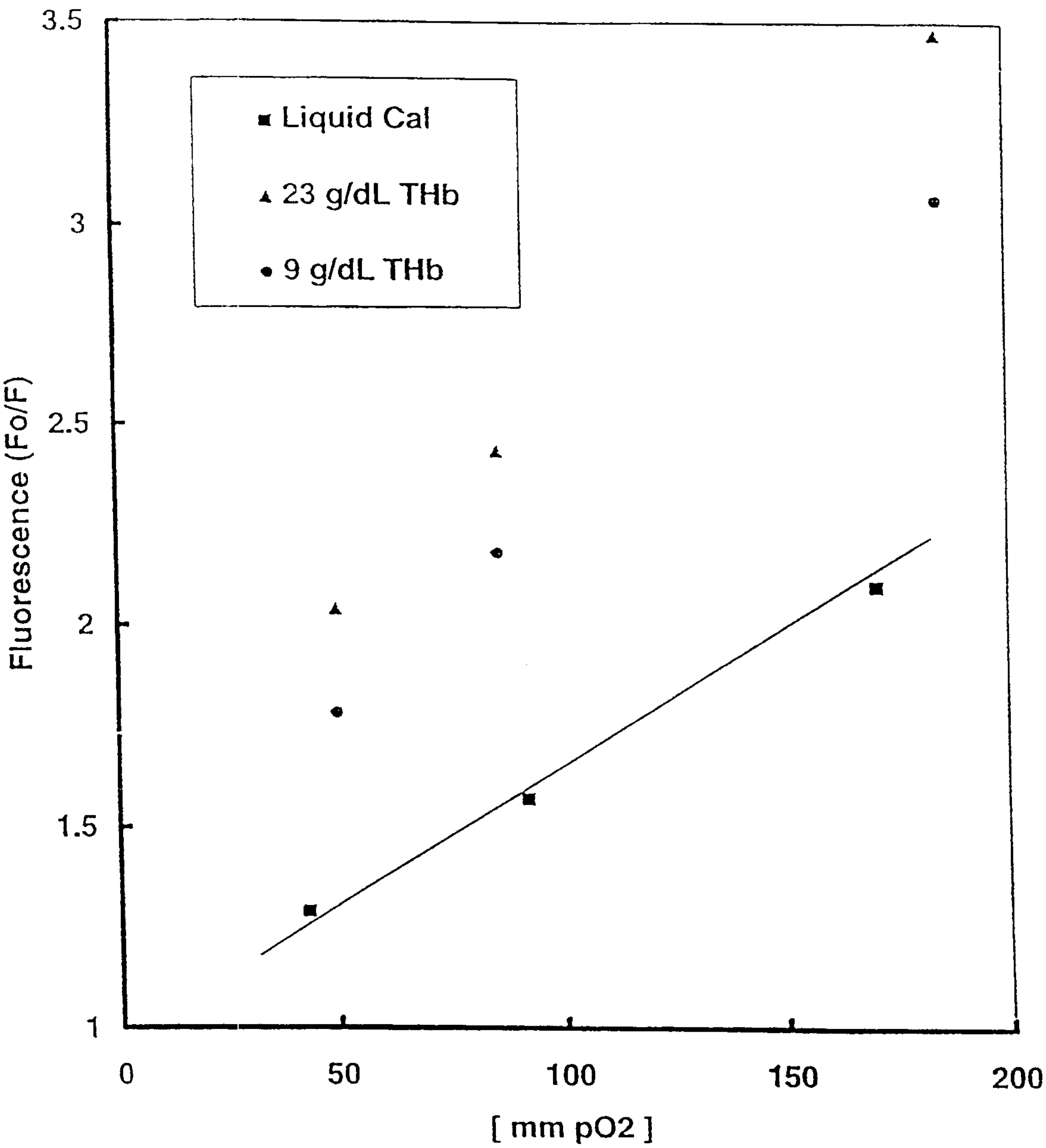
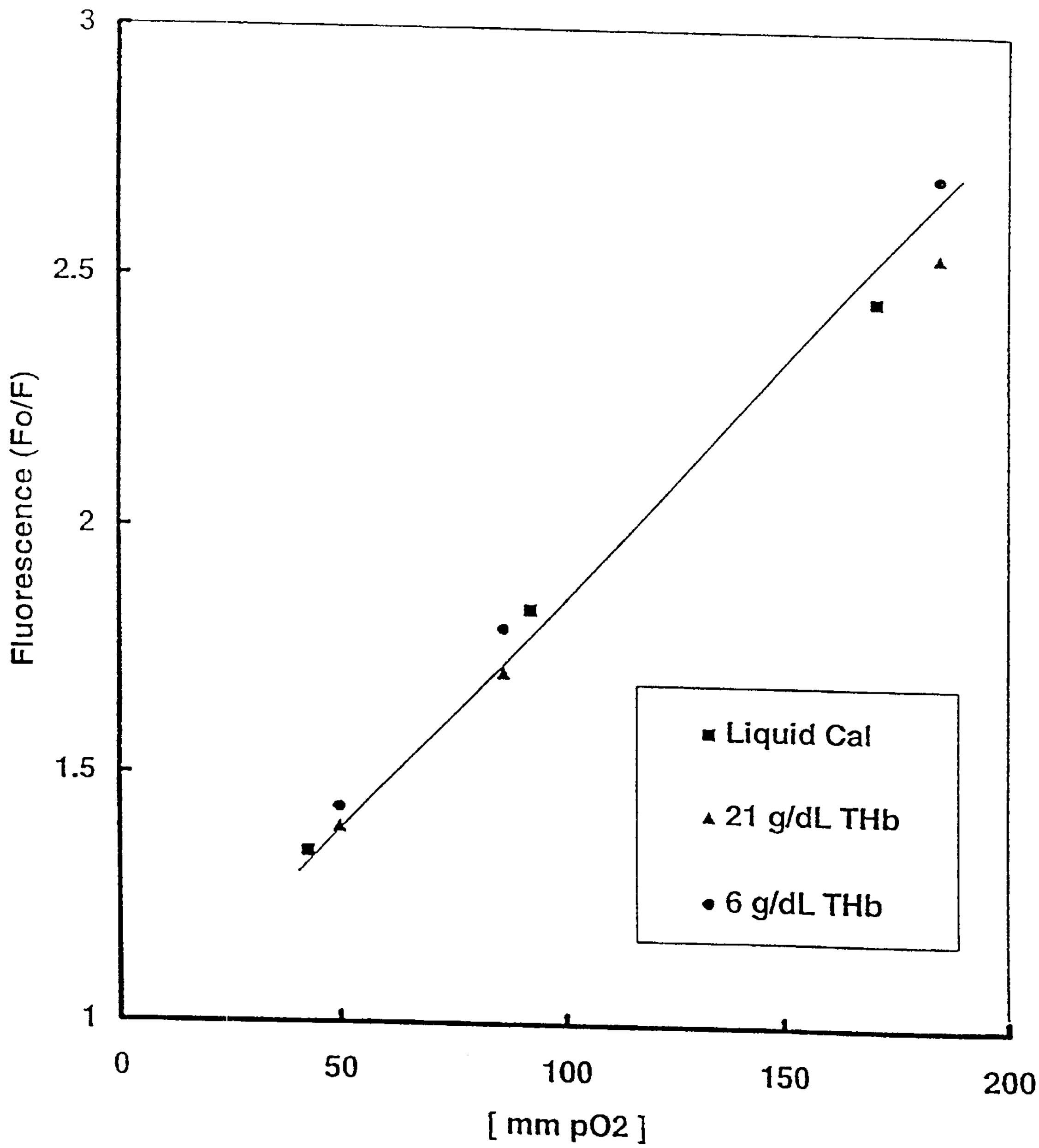

3.5
3
2.5
2
1.5
1
Fluorescence (Fo/F)
Liquid Cal
23 g/dL THb
9 g/dL THb
0
50
100
150
200
[mm pO2]

FIG. 3A



3
2.5
2
1.5
1
0
50
100
150
200
Fluorescence (Fo/F)
[mm pO2]
Liquid Cal
21 g/dL THb
6 g/dL THb

FIG. 3B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 99/01446

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/52 G01N31/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 584 721 A (BOEHRINGER MANNHEIM GMBH) 2 March 1994 (1994-03-02) column 3, line 29 -column 4, line 58 column 8, line 21 - line 23 column 9, line 48 - line 52; figure 1 ---	1-7, 10-18,21 8,9,19, 20
X Y	US 4 255 384 A (KITAJIMA MASAO ET AL) 10 March 1981 (1981-03-10) column 2, line 49 -column 3, line 20 column 4, line 57 -column 5, line 8 column 6, line 59 - line 68 --- -/--	1-7, 10-18,21 8,9,19, 20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
10 December 1999	20/12/1999

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Inter nal Application No
PCT/IB 99/01446

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5 609 823 A (HARTTIG HERBERT ET AL) 11 March 1997 (1997-03-11) column 2, line 4 - line 10 column 3, line 30 - line 43	1-7, 10-18,21 8,9,19, 20
A	US 4 248 829 A (KITAJIMA MASAO ET AL) 3 February 1981 (1981-02-03) column 5, line 3 - line 25	1-7, 10-18,21
Y	WO 97 37210 A (CHIRON DIAGNOSTICS CORP ;SLOVACEK RUDOLF E (US); SULLIVAN KEVIN J) 9 October 1997 (1997-10-09) abstract; examples 4-7	8,9,19, 20

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 99/01446

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
EP 0584721	A	02-03-1994	DE	4227665	A	24-02-1994
			AT	160221	T	15-11-1997
			DE	59307664	D	18-12-1997
			ES	2109404	T	16-01-1998
			JP	2520220	B	31-07-1996
			JP	6235695	A	23-08-1994
US 4255384	A	10-03-1981	JP	1355882	C	24-12-1986
			JP	55026428	A	25-02-1980
			JP	61022905	B	03-06-1986
			JP	1355883	C	24-12-1986
			JP	55026429	A	25-02-1980
			JP	61022906	B	03-06-1986
			DE	2932973	A	28-02-1980
US 5609823	A	11-03-1997	DE	4326339	A	09-02-1995
			EP	0637749	A	08-02-1995
			JP	2610109	B	14-05-1997
			JP	7077528	A	20-03-1995
			US	5679311	A	21-10-1997
US 4248829	A	03-02-1981	DE	2949747	A	19-06-1980
WO 9737210	A	09-10-1997	US	5863460	A	26-01-1999
			AU	2039597	A	22-10-1997
			EP	0891540	A	20-01-1999